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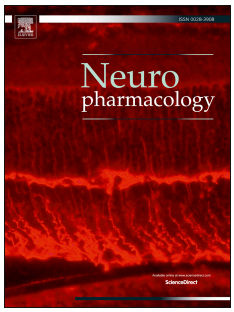

Antidepressant-induced increase in GluA2 expression does not translate in changes of AMPA receptor-mediated synaptic transmission at CA3/CA1 synapses in rats

Elisabetta Gerace, Lorenzo Polenzani, Maurizio Magnani, Elisa Zianni, Gabriella Stocca, Fabrizio Gardoni, Domenico E. Pellegrini-Giampietro, Renato Corradetti

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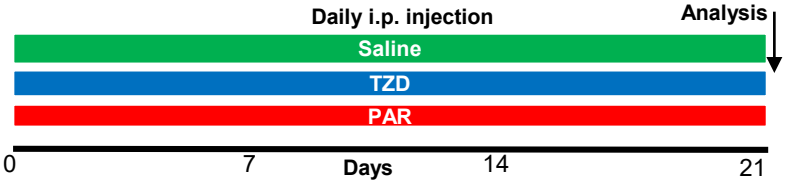
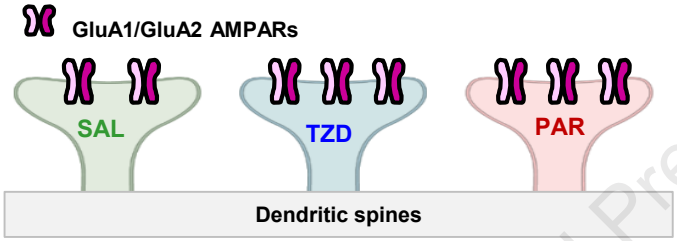
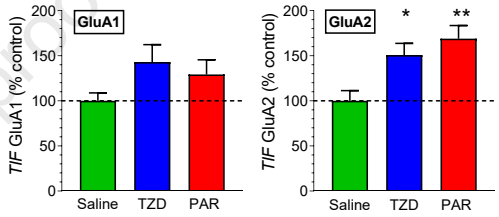
Renato Corradetti: Conceptualization, Formal analysis, Supervision, Project administration, Writing Original Draft, Writing - Review & Editing , Resources, Funding acquisition

Saline
Trazodone (10 mg/kg)
Paroxetine (10 mg/kg)

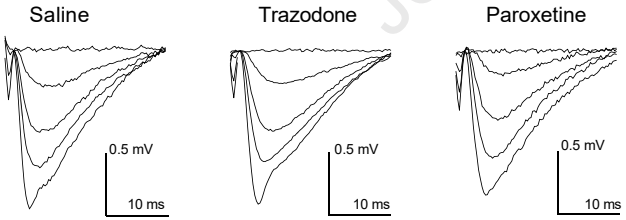


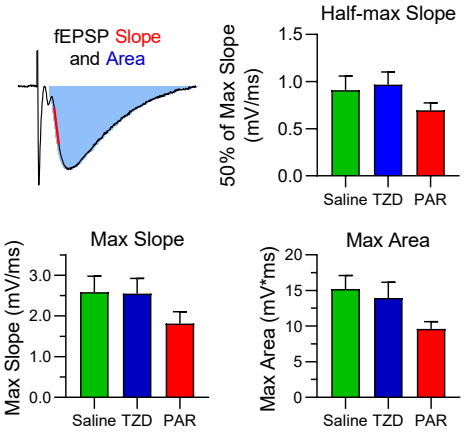
Increase in hippocampal GluA2 AMPAR subunit expression





No change in fEPSP responses at CA3/CA1 synapses





Antidepressant-induced increase in GluA2 expression does not translate in changes of AMPA receptor-mediated synaptic transmission at CA3/CA1 synapses in rats.

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Highlights:

- Chronic antidepressant treatment changes AMPAR subunit expression in rat brain
- Chronic trazodone or paroxetine increased hippocampal GluA2 subunit expression
- Excitatory basal neurotransmission in CA1 was studied ex-vivo in hippocampal slices
- Both antidepressants did not significantly affect AMPAR-mediated responses in CA1
- Increased GluA2 subunits do not translate in facilitation of basal CA1 transmission

Keywords:

Trazodone, paroxetine, AMPA receptors, hippocampus, chronic treatment, IEM 1460

Abstract

Chronic treatment with serotonin selective reuptake inhibitors or tryciclic antidepressant drugs in rodents has been shown to increase the expression of GluA1 and/or GluA2 AMPA receptor (AMPAR) subunits in several brain areas, including the hippocampus. These changes in AMPAR composition have been suggested to result in increased glutamatergic neurotransmission and possibly underlie enhanced hippocampal synaptic plasticity through the increased availability of calcium-permeable AMPARs, specifically at CA3/CA1 synapses. However, the possibility that chronic treatment with antidepressants actually results in strengthened glutamatergic neurotransmission in CA1 has poorly been investigated. Here, we studied whether chronic treatment with the multimodal antidepressant drug trazodone mimicked the effect of paroxetine on the expression of AMPAR subunits in male wistar rat hippocampus and whether these drugs produced a parallel facilitation of field excitatory postsynaptic potentials (fEPSP) responses evoked by activation of CA3/CA1 synapses in dorsal hippocampal slices. In addition, we investigated whether the quality of glutamatergic AMPARs involved in basal neurotransmission was changed by altered subunit expression, e.g. leading to appearance of calcium-permeable AMPARs. We found a significant increase in GluA2 subunit expression following treatment with trazodone or paroxetine for twenty-one days, but not after seven-days treatment. In contrast, we did not find any significant changes in fEPSP responses supporting either a facilitation of glutamatergic neurotransmission in basal conditions or the appearance of functional calcium-permeable AMPARs at CA3/CA1 pyramidal neuron synapses. Thus, neurochemically-detected increases in the expression of AMPAR subunits cannot directly be extrapolated in increased number of functioning receptors and/or facilitated basal neurotransmission.

1. Introduction

Studies in post-mortem cerebral tissue of depressed patients indicate that changes in the expression of AMPA receptors (AMPARs) in several brain areas are associated with mood disorders (Beneyto et al., 2007; Duric et al., 2013). Thus, AMPARs have been proposed to be involved in major depression and in the actions of classical and novel, fast acting, antidepressant drugs (Alt et al., 2006; Duric et al., 2013). However, it is still unclear whether these changes are a consequence of pathophysiological alterations due to the disorder or whether they are more directly involved in the development of depression and therefore represent a primary target for antidepressant treatments.

Interestingly, in rodents, chronic treatment with various antidepressant drugs has been shown to increase the expression of GluA1 and/or GluA2 subunits of AMPARs in several brain areas, including hippocampus. For instance, chronic treatment with antidepressant drugs that inhibit serotonin reuptake, such as paroxetine, imipramine or desipramine, significantly increase the expression of both the AMPAR subunits GluA1 and GluA2/GluA3 in the rat hippocampus (Martinez-Turrillas et al., 2002; Martínez-Turrillas et al., 2005, 2007). Moreover, increase in GluA1 and GluA2 levels were also observed in hippocampus and nucleus accumbens of mice treated for 30 days with maprotiline (Tan et al., 2006) and in cortex of rats following 28 days treatment with fluoxetine, where a selective increase in GluA2 but not GluA1 in synapses was found (Ampuero et al., 2010).

Altogether, the present knowledge on antidepressant drug mechanism(s) of action indicates that changes in hippocampal AMPAR expression and/or trafficking might represent a therapeutic commonality in cellular response(s) to treatments of affective disorders. This suggests that newly synthesised AMPARs, if incorporated in synapses, could facilitate basal glutamatergic neurotransmission and plasticity, resulting in functional effects similar to ampakines, that have been recently proposed in therapy of depression (Freudenberg et al., 2015; Kadriu et al., 2021).

Functional AMPARs are tetramers typically formed by heteromeric combination of two out of the four possible subunits (GluA1 - GluA4), mostly localised in the postsynaptic density and synapse-specifically expressed onto interneurons (Szabo et al., 2012; Lalanne et al., 2018). At hippocampal CA3/CA1 synapses, the large majority (~80%) of AMPARs expressed are GluA1/GluA2 (Lu et al., 2009). According to their subunit composition, AMPARs have different properties and in particular, GluA2 subunit regulate AMPAR assembly and ionic selectivity (Greger et al., 2007; Liu and Zukin, 2007; Milstein and Nicoll, 2008). Therefore, a relative decrease in GluA2 subunit increases calcium permeability of AMPAR channels. In addition, under some circumstances, unedited GluA2 subunits may be assembled in functional AMPARs and retain calcium permeability (see in Wright and Vissel, 2012). Thus, modified subunit composition of AMPARs may alter the quality of AMPAR expressed, leading to the appearance of calcium-permeable AMPARs (CP-AMPARs) as shown to happen with

cocaine abuse/treatment (Bellone and Lüscher, 2006; Clem and Huganir, 2010). However, in spite of changes in the expression of AMPAR subunits produced by several antidepressant drugs, the possible consequences of chronic treatment with these medicines have scarcely been investigated both on basal synaptic excitatory transmission (O'Connor et al., 1993) and synaptic plasticity in the hippocampus (Marchetti et al., 2010; Rubio et al., 2013; Popova et al., 2017).

The aim of the present study was to investigate whether increased expression of GluA1 and/or GluA2 subunits produced by chronic treatment with antidepressant drugs translates in changes in basal glutamatergic transmission at CA3/CA1 synapses in the dorsal hippocampus. To this purpose, we selected paroxetine, a serotonin selective reuptake inhibitor (SSRI) for which the ability to change AMPAR subunit expression and trafficking has previously been established (Martinez-Turrillas et al., 2002; Martínez-Turrillas et al., 2005, 2007) and trazodone, a multimodal antidepressant drug that, in addition to its inhibitory activity at cell membrane 5-HT transporter (SERT), is a competitive ligand at 5-HT1A, 5-HT2A, 5-HT2C receptors and α1-adrenoceptors for which it displays considerable affinity in human and rodent brain tissue (Cusack et al., 1994; Owens et al., 1997).

Here, we investigated whether chronic treatment for three weeks with trazodone and paroxetine modifies the expression of AMPAR subunits in the rat hippocampus and whether changes in AMPAR composition lead to changes in fEPSP responses evoked by activation of CA3/CA1 synapses in the dorsal hippocampus.

2. Materials and methods

2.1. Reagents

Trazodone hydrochloride and paroxetine hydrochloride were provided by ACRAF. IEM-1460 was purchased from Tocris Cookson (Bristol, UK), D-(-)-2-amino-5- phosphonopentanoic acid (APV) and 6-nitro-7-sulphamoylbenzo(f)quinoxaline hydrochloride (NBQX) were purchased from Ascent Scientific (UK). Salts used for solutions (aCSF) were from Merck Chemicals and sterile saline for use in humans or apyrogen water was used to dissolve drugs for injection.

2.2. Animals

Male Wistar rats (Harlan, Italy) were group-housed, with two in each cage. All animals were kept in a temperature- and humidity-controlled environment with a light:dark cycle of 12:12 h (lights on at 8:00 a.m.), with food and water available ad libitum. All animal manipulations were carried out according to the European Community guidelines for animal care, approved by the Committee for Animal Care and Experimental Use of the University of Florence and authorized by the Italian Ministry of Health (Auth: 206/2010-B).

2.3 Treatments.

Male rats weighing 180 ± 2 g at the beginning of the treatment were treated for 7 or 21 days with saline (approximately 0.5-0.7 ml, i.p.), trazodone (10 mg/kg, i.p./day dissolved in saline) or paroxetine (10 mg/kg, i.p./day dissolved in distilled water). Twentyfour hours after the final injection, animals were sacrificed, the hippocampi were rapidly removed for either neurochemical assay of GluA1 and GluA2 receptor subunit expression on TIF extracts (see below) or electrophysiological experiments in vitro. Neurochemical and electrophysiological experiments were carried out in parallel and treatments were interleaved to minimize clustering. The treatment schedule was ideated so that the day of mid-treatment coincided for all groups (+/- 1 day).

2.4 Subcellular fractionation

Total homogenates and Triton-insoluble postsynaptic fractions (TIF) were obtained as previously described (see (Gardoni et al., 2001); (Gardoni et al., 2006)). In brief, rat hippocampi were manually homogenized twice in lysis buffer (Sucrose 0.32 M, Hepes 1 mM, Magnesium Chloride 1 mM, Sodium carbonate 1 mM). To obtain TIF, homogenates were centrifuged at 1000xg for 5 min at 4°C. The resulting supernatants were pooled and centrifuged 13,000xg for 15 min at 4°C. Pellets were then resuspended in 1 mM Hepes and ultra-centrifuged 100,000xg for 1 hr at 4°C. Precipitates were dissolved, incubated for 15 min in 150 mM potassium chloride, 0.5% Triton and ultra-centrifuged again 100,000xg for 1 hr at 4°C. The final pellets (TIF) were homogenized with a glass-glass potter

in Hepes 20 mM buffer. All purification steps were performed in the presence of protease and phosphatase inhibitor cocktails (Roche Diagnostics, Monza, Italy).

2.5 Western blotting analysis

Western blot assays were performed in the total homogenate and TIF as previously described (see (Piva et al., 2018); (Gerace et al., 2020). After separation by SDS-PAGE on an 7% gel under denaturing conditions, the proteins were transferred onto a nitrocellulose membrane. Membranes were blocked with I-block solution (Invitrogen, Thermo Scientific, Milan, Italy) and incubated overnight with primary antibodies (anti-GluA1 antibody, Cell Signaling, BK131855, 1:500; anti-GluA2 antibody, Neuromab #75-002, 1:500; anti-Tubulin antibody, Sigma Aldrich, T9026, 1:5,000), followed by incubation with horseradish peroxidase-linked anti-rabbit or anti-mouse IgG antibody (1:5,000, Biorad, Hercules, CA, USA) in TBS containing 0.1% Tween-20 at room temperature for 1h. Finally, proteins were detected using an electrochemical luminescence (ECL) kit (Clarity Western ECL substrate, Bio-Rad, Hercules, CA, USA or LiteAblot TURBO, Euroclone, Milan, Italy). Quantification was performed using ImageJ software, and each protein was normalised on the corresponding Tubulin band run in the same gels.

2.6. Preparation of hippocampal slices

Electrophysiological extracellular recordings were carried out in hippocampal slices taken from a subset of rats, treated as described above for 7 or 21 days with either saline (n=4,7), paroxetine (n=4,6) or trazodone (n=4,7). Slice preparation was carried out as previously described (Mlinar et al., 2006). Animals were euthanized, and hippocampi were rapidly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF), which contained the following (in mM): NaCl, 126; KCl, 2; KH2PO4, 1.25; NaHCO3, 26; MgSO4, 1.5; CaCl2, 2; D-glucose 10. The solution was bubbled with a 95% O2 / 5% CO2 gas mixture (pH 7.4). Transversal hippocampal slices of 400 μM nominal thickness were cut with a McIlwain tissue chopper (Gomshall, U.K.) and kept at least 1.5 h at room temperature until recording. Before transferring to the recording chamber, a single slice was temporarily transferred to a Petri dish, where the CA1 region was disconnected from the CA3 region by a surgical cut. The slice was then placed on a nylon mesh, completely submerged in a recording chamber and continuously superfused (2.0 - 2.2 ml min-1) with oxygenated ACSF at 32 – 33 °C. Slices were incubated for 15 minutes in the recording chamber before initiating electrical stimulation that was continuous throughout the experiment. All drugs were applied via bath perfusion.

2.7. Electrophysiology recordings

Synaptic responses of CA1 pyramidal neurons were elicited by stimulation of the Schaffer collateral / commissural pathway. Stimulation pulses (80 μs duration; 10-15 s interpulse interval), triggered by a PC controlled by p-Clamp software (Molecular Devices) were delivered by a stimulus isolation unit (DS2, Digitimer, Welwyn Garden City, U.K.) through concentric bipolar nichrome electrodes (FHS, Bowdoin, ME, U.S.A). Field potentials were recorded with glass electrodes (filled with 150 mM NaCl, 2 - 10 MΩ resistance) placed in the distal third of the stratum radiatum to record field excitatory postsynaptic potentials (fEPSP). The distance between recording electrodes and the stimulating electrode was 200-300 μm Recorded potentials were amplified with Neurolog NL 104 amplifiers (Digitimer), digitized with DIGIDATA 1340 interface (Molecular Devices; sampling rate 50 kHz) and stored in a PC for off-line analysis. fEPSP was determined as the slope of the initial falling phase of the response recorded in the stratum radiatum. In addition, the integral of the response was measured and termed fEPSP area (see Fig. 2).

In each preparation, the stimulus-response relationship was obtained by stepwise increase of the stimulating current (20-240 μA) in baseline conditions before the application of drugs. Then, the stimulus intensity of test pulses was set to evoke a fEPSP that had a slope ~ 40 % of the maximum in the preparation and was held constant throughout the remainder of the experiment. At least 10 min of stable responses were used to generate the baseline values before drug application.

A small set of patch-clamp recordings were carried out in hippocampal slices of naive young (12-15 days-old) wistar rats to test effectiveness of IEM 1460 concentration (50 μM) used in extracellular experiments as described in Suppl Fig. 4.

2.8. Pharmacology

After obtaining an adequate baseline 20 μM D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5) was superfused to block the NMDA component of fEPSPs and to isolate the AMPA receptor-mediated response of excited CA1 pyramidal neuron dendrites. IEM 1460 (50-90 μM) was added after the attaining of a steady state response to D-AP5 (10-20 min) and was applied for at least 20 min. In most experiments, at the end of experiments, the selective AMPA receptor antagonist NBQX (10 μM) was added to the superfusion fluid to estimate the residual non NMDA component of the evoked field potential.

2.9.Statistical analysis

Data were analyzed using Clampfit 10 (Molecular Devices) and Prism 8 software (GraphPad Software, San Diego, CA, USA). For each experiment, the relationship between the amplitude of the afferent volleys and the slope of fEPSPs was analyzed by linear regression and comparison of the slope of the resulting regression line was used for initial assessment of the effect of the different

chronic treatments on basal neurotransmission. Then, the corresponding stimulus-responses relationship was plotted on semilogarithmic scale and data fitted using four-parameter logistic equation. The calculated maximal response was used for statistical comparison amongst groups. For clearer graphical representation of data grouped by treatment, the curves were normalized to the respective maximal response. For each experiment the stimulus which evoked the half-maximal response (EStim50) and the slope of the curve (nH) were also calculated. For statistical comparison, the steady-state values were computed by averaging 7-11 consecutive responses obtained over 5 min period immediately before drug application (baseline value) and at the end of in vitro drug application. Unless otherwise stated, data were expressed as the mean ± SEM and were analyzed statistically with the use of one-way ANOVA, followed by Tukey or Dunnett post-hoc tests, by Wilcoxon Signed Rank Test, one sample t test, two tails versus no effect in Fig. 4, as appropriate. A value of $p < 0.05$ was considered significant.

3. Results

3.1 *GluA1/GluA2 receptor subunit expression in hippocampal TIF extracts from antidepressant treated rats*

Figure 1 illustrates the changes in the expression of GluA1 and GluA2 in total homogenates and in synaptic membrane-enriched TIF homogenates, produced by twenty one days-treatment with trazodone or paroxetine. No significant changes in the expression of both GluA1 or GluA2 were found in total homogenates ($F_{(2, 12)}$ = 0.005, p=0.9946 and $F_{(2, 12)}$ = 3.221, p=0.0759, respectively; one-way ANOVA; Fig. 1A). Conversely, in TIF homogenates, the expression of GluA2 was significantly increased after chronic treatment with either trazodone (51 ± 13 %, mean ± s.e.m; n=5) or paroxetine (69± 15 %, mean ± s.e.m; n=5) compared to saline-treated rats ($F_{(2,12)}$= 7.592, p=0.0074; p<0.05 and p<0.01, respectively; one-way ANOVA followed by Dunnett's test). GluA1 showed smaller increases in expression that did not reach statistical significance ($F_{(2, 12)}$ = 2.019; p=0.1755; Fig. 1B). Shorter treatments (seven days) with trazodone or paroxetine did not significantly affect GluA1 and GluA2 subunit expression, both in TIF and total homogenates (Suppl. Fig. 1).

3.2 *Glutamate-mediated basal neurotransmission at CA3/CA1 synapses is not affected by treatments*

In parallel with neurochemical experiments that indicated an increase in the expression of GluA2 AMPAR subunits, we studied the effects of trazodone or paroxetine on basal excitatory CA3/CA1 synaptic transmission in ex vivo hippocampal slice preparations. Specifically, we investigated the possible appearance of a calcium-permeable AMPA receptor (CP-AMPA)-mediated component of neurotransmission using in vitro pharmacological tools. To this aim, we tested the sensitivity of fEPSPs to the selective NMDA receptor antagonist APV and to the selective blocker of CP-AMPA receptors IEM 1460 (Fig. 2). In addition, stimulus-response curves (SRCs) of fEPSPs recorded prior to in vitro application of drugs in slices from chronically-treated rats were recorded to study whether treatments had changed synaptic responses in basal conditions.

In 21 days-treated animals the relationships between the afferent volley and the fEPSP slope were fairly linear and to compare the results in different experiments we calculated the slope of the regression line of responses. This analysis did not reveal any significant differences (F (2, 28) = 1.615; p =0.217; ANOVA) amongst treatment groups. The slopes of the volley-response relationships were (mean ± SEM): Saline: 4.56 ± 0.53 [(mV/ms)/mV; n=11]; Trazodone 5.4 ± 0.93 [(mV/ms)/mV; n=11]; Paroxetine 3.36 ± 0.83 [(mV/ms)/mV; n=9]. Further analysis of basal SRCs of fEPSPs in slices from trazodone or paroxetine-treated rats revealed no differences from those obtained from saline-treated animals, indicating that neither trazodone nor paroxetine treatment affected the basal neurotransmission strength. Thus, no statistical differences were found for all parameters of stimulus-

response curves relative to fEPSP slope and area, such as the maximal fEPSP, the stimulus intensity producing half-maximal response and the nH coefficient of stimulus-response curve slope (Fig. 3). Indeed, one-way ANOVA analysis revealed no differences amongst in vivo treatments in the maximal fEPSP slope ($F(2,28)=1.337$, $p=0.2788$) and area ($F(2,28)=2.181$, $p=0.1101$). No differences were also found in parameters characterizing the curves such as the stimulus intensity producing half-maximal response for fEPSP slope ($F(2,28)=0.5418$, $p=0.5877$) and fEPSP area ($F(2,28)=0.8918$, $p=0.4212$) as well as in nH, the coefficient of stimulus-response curve slope, for both fEPSP slope ($F(2,28)=1.295$, $p=0.2897$) and fEPSP area ($F(2,28)=2.667$, $p=0.0870$). Moreover, the responses to low stimulus strength did not differ amongst groups of treatment (Suppl. Fig. 2)

3.3 *In vivo treatments did not produce the appearance of calcium-permeable AMPA receptors at CA3/CA1 synapses*

In order to examine the contribution of NMDA and CP-AMPA receptors, synaptic responses were sequentially tested for their sensitivity to APV (20 μM, 15-25 min application) and IEM 1460 (50 μM, 20 min) in hippocampal slices taken from animals treated for seven (Suppl Fig 3) or twenty-one days with saline, trazodone or paroxetine (Fig. 4,5). The effectiveness of IEM 1460 in blocking CP-AMPA receptors was confirmed using whole-cell patch-clamp recordings from non pyramidal CA1 neurons, known to physiologically express CP-AMPA receptors (Szabo et al., 2012; Lalanne et al., 2018) Suppl. Fig. 4), in untreated rats.

Application of APV in slices obtained after twenty-one days of treatment with trazodone, paroxetine or saline did not affect fEPSP slope while it produced a decrease of fEPSP area, as expected from the block of the NMDA receptor-mediated late component of EPSPs (Fig. 4). Although the effect was small (~10% overall), the decrease in fEPSP area produced by APV respective to the pre-drug baseline resulted significant in all groups (saline: -12.17 ± 1.423 %, $p=0.0078$, $n=7$; trazodone: -9.81 ± 1.4649 %, $p= 0.0078$, $n=7$; paroxetine: -6.6 ± 2.4771 %, $p= 0.0391$ $n=7$; Wilcoxon Signed Rank Test, one tail). IEM 1460, applied in the presence of APV, did not produce further significant reduction of fEPSP area in all groups (saline: 0.79 ± 3.73 %, $n=7$; $p=0.500$; trazodone: -1.74 ± 1.83 %, $n=8$, $p=0.1875$; paroxetine: +5.05 ± 2.57 %, $n=7$, $p=0.0781$; Wilcoxon Signed Rank Test, one tail), confirming that both trazodone and paroxetine did not induce the appearance of functional CP-AMPA receptors at CA3/CA1 pyramidal neuron synapses. Figure 5 illustrates the time-course of the responses to the sequential application of APV and IEM 1460 in the three groups of treated rats. In all preparations, NBQX, added to APV and IEM 1460 at the end of the experiment, abolished stimulus-evoked fEPSPs (not shown; see e.g. Fig. 2).

4. Discussion

The objectives of the present study were to investigate whether chronic treatment with trazodone or paroxetine for three weeks leads to modification of AMPAR subunits, and most importantly, whether these qualitative composition of AMPA channels could translate into increased CA3/CA1 hippocampal neurotransmission in basal conditions and/or in parallel changes in permeability (CP-AMPA) of AMPAR.

In our investigation, we have selected two widely prescribed antidepressant drugs, i.e. paroxetine, a SSRI reportedly able to increase the expression of AMPAR GluA1 and GluA2 subunits and trazodone as representative of the class of multimodal antidepressants (Stahl, 2009). The pharmacological effects of trazodone have been extensively studied *in vitro* and *in vivo* (Ghanbari et al., 2010; Montalbano et al., 2019), as well as the drug's clinical effects (Stahl, 2009); (Settimo and Taylor, 2018). Nevertheless, the full spectrum of trazodone's actions in the CNS is not completely established and, in particular, the possibility that its multiple pharmacological effects modify glutamatergic excitatory neurotransmission has not been explored, yet. Our neurochemical results demonstrated that chronic treatment with both antidepressant drugs causes a significant increase in the expression of the AMPAR subunit GluA2 and a trend to increase of GluA1 in hippocampal postsynaptic compartment. Previous work showed that chronic treatment with antidepressants that inhibit serotonin reuptake, such as paroxetine, imipramine or desipramine, significantly increase the expression of GluA1 and GluA2/GluA3 AMPAR subunits in the rat hippocampus (Martinez-Turrillas et al., 2002; Martínez-Turrillas et al., 2005, 2007).

Our results showed that chronic treatment with trazodone or paroxetine produced a clear increase in GluA2 subunits whereas GluA1 expression was not increased to a similar extent. The discrepancy with previous findings by Martinez-Turrillas and Colleagues may reside in the fact that in these reports AMPA receptor subunits were evaluated in total membrane fractions, thus comprising synaptic, extrasynaptic and intracellular fractions, using less specific GluA2 antibodies. Our analysis was instead conducted on the highly purified synaptic fraction, localized at the PSD level. On the other hand, our results are in agreement with the selective increase in expression of GluA2 observed in rat hippocampus (Rubio et al., 2013) and in hippocampus and cortex (Ampuero et al., 2010) following 28 days treatment with fluoxetine.

Collectively, these data suggest that changes in the expression of AMPAR subunits and in particular of GluA2, represent a common step involved in antidepressant action of these drugs.

A recurrent interpretation of functional consequences of these changes is that increased expression of GluA1 and/or GluA2 would result in increased glutamatergic neurotransmission. Indeed, should such changes be indicative of an increase in the expression and membrane exposure of AMPARs, the

glutamatergic neurotransmission should result enhanced in the hippocampus. Moreover, in the case that GluA2 subunit would be incorporated in receptors in its unedited form, the additional AMPARs at synaptic level could theoretically be permeable to calcium (Wright and Vissel, 2012) and lead to detectable changes in the "quality" of ionic conductance comprised in the EPSPs. It is therefore conceivable that, if new AMPARs are stably exposed at synaptic connections, the basal neurotransmission would be detectably enhanced both *in vivo* and in *ex vivo* measurements in hippocampal slices.

However, the number of studies specifically investigating the consequences of chronic antidepressant drug treatment on hippocampal basal synaptic responses is limited and with often contrasting results. For instance, O'Connor and Colleagues by recording electrically evoked fEPSP in CA1 from pentobarbital anaesthetized rats *in vivo* showed a marked decrease (>50 %) in evoked potential amplitude after chronic treatment with imipramine (O'Connor et al., 1993). In contrast, *in vivo* recording of electrically evoked fEPSP in the dentate gyrus of rats treated with desipramine or mianserin for one week showed no changes in basal stimulus-response relationship for both EPSP slope and population spike amplitude (Levkovitz et al., 2001). Moreover, in anaesthetized rats, treatment with fluoxetine for two weeks produced an increase in basal stimulus-response relationship for both EPSP slope and population spike amplitude in the dentate gyrus (Stewart and Reid, 2000), a result not confirmed in un-anaesthetized, freely moving rats treated with fluoxetine for up to six weeks (Keith et al., 2007).

Further information on the effects of chronic antidepressant drug treatment can also be derived from *ex vivo* experiments in hippocampal slices. The few available studies, however, are restricted to the effects of *in vivo* chronic treatment with fluoxetine and addressed to the consequences on synaptic plasticity at CA3/CA1 synapses, so that changes in basal synaptic neurotransmission were not thoroughly investigated. Thus, Rubio and colleagues found that chronic treatment with fluoxetine strengthen basal stimulus-response relationship for fEPSP and occlude long-term potentiation in rats (Rubio et al., 2013). Interestingly, immunostaining showed an increase in GluA2, but not in GluA1, AMPAR subunits. On the other hand, Popova and Colleagues reported no changes in basal CA3/CA1 neurotransmission and increased LTP in fluoxetine treated mice (Popova et al., 2017).

Our investigation was therefore designed to directly test the possible relationship between the increase in the expression of GluA2 and a persistent increase in strength of AMPAR signalling at CA3/CA1 synapses by accurately measuring the stimulus response relationship of fEPSPs in basal conditions with particular care in determining the maximal response attainable, which could be indicative of an increase in the expression/exposure of an enhanced number of AMPARs. Furthermore, we investigated whether CP-AMPA receptors would participate in the EPSP generation. In particular,

we measured the fEPSP area that reports changes in the late part of fEPSPs and is appropriate for the study of NMDA component and for detecting the possible appearance of CP-AMPA receptors.

Unexpectedly, we did not find any detectable changes in fEPSP responses supporting the appearance of new AMPARs and in particular of functional CP-AMPA receptors at CA3/CA1 pyramidal neuron synapses. Thus, GluA2 overexpression was not accompanied by facilitation of basal synaptic transmission at CA3/CA1 synapses, but we cannot exclude that the increase in GluA2 could be involved in enhanced synaptic plasticity in the hippocampus.

In fact, reduction in hippocampal plasticity has been found in depressed patients (Duric et al., 2013) and in animal models of depression (Yuen et al., 2012; Duric et al., 2013) accompanied by reduced levels of GluA1) and, more importantly, that antidepressant treatment produces a recovery of synaptic plasticity in models of depression.

The role of GluA2 containing or GluA2 lacking-AMPARs in plasticity is still debated. Adesnik and Nicoll (Adesnik and Nicoll, 2007) showed that insertion of GluA2-lacking, calcium-permeable AMPARs is not required for LTP, although the intracellular AMPAR pool is needed for LTP, regardless the subunit composition (Granger et al., 2013). Therefore, it is possible that GluA2 overexpression reports an increase in intracellular AMPARs available for plasticity.

On the other hand, it should not be overlooked that a durable increase in CP-AMPA, by increasing calcium entry, has been shown to be a neurotoxic factor. In general, prolonged decrease in surface GluA2-containing AMPARs in favour of GluA1 homomeric AMPAR assembly and the subsequent appearance of CP-AMPARs has been associated with pathological states (Bennett et al., 1996; Hideyama and Kwak, 2011). However, GluA2 editing is crucial for preventing the entry of calcium through the receptor pore and unedited GluA2 containing AMPARs have been shown to be calcium permeable (see in Wright and Vissel, 2012). Therefore also a selective increase in unedited GluA2 could lead to CP-AMPA receptor membrane exposure. Changes in functional expression of CP-AMPARs in animal models have been shown to play a role in the effects of substances of abuse, such as cocaine (Mameli et al., 2011; Pascoli et al., 2011; Mills et al., 2017)and ethanol (Gerace et al., 2021) and persistent, unphysiological, entry of calcium has been described as a causal factor for cell death of hippocampal CA1 pyramidal neurons in models of toxicity (Anzai et al., 2003; Gerace et al., 2014) and in pathological conditions including global ischemia (Pellegrini-Giampietro et al., 1997; Gorter et al., 1997; Noh et al., 2005).

In our experimental conditions, neither trazodone nor paroxetine induced the formation and insertion of functional CP-AMPARs in hippocampal synapses, indicating that long-term treatment with these antidepressant drugs is unlikely to produce neuron damage.

It deserves mention that the present work has been focussed on neurotransmission at CA3/CA1 synapses, which prevents uncritical generalization of our results to brain pathways different from CA3/CA1. Furthermore, the neurochemical assays were performed in the whole hippocampus, which does not rule out the possibility that the increase in expression of GluA2, and in particular of unedited GluA2, is localized in hippocampal inhibitory GABA neurons, where CP-AMPA receptors are physiologically expressed (Albuquerque et al., 1999; Zinchenko et al., 2021). Similarly, we did not investigate a possible dorsoventral distribution of GluA2 overexpression and the electrophysiological analysis were limited to dorsal hippocampus.

Finally, we have selectively studied neurotransmission at CA3/CA1 synapses, but within the hippocampus excitatory synapses of other pathways could be the site of increased GluA2 subunit expression and functional changes following antidepressant drug treatment. For example, Kallarackal and Colleagues found that within area CA1, the effects of fluoxetine were different in stratum radiatum and stratum lacunosum-moleculare (Kallarackal et al., 2013) of chronically stressed rats.

Notwithstanding these considerations, the emerging result is that CA3/CA1 synapses that represent the major efferent pathway from the hippocampus, are not persistently facilitated.

A further important consideration is that this study investigated the pharmacological effects of antidepressant drugs in normal "non depressed" animals and therefore it cannot be concluded that GluA2 subunit expression changes are not meaningful in depression. Under this perspective, further investigation in animal models of depression is warranted to establish whether long-term antidepressant drug treatment could effectively counteract or prevent possible alterations in brain excitatory neurotransmission correlated to changes in the expression of AMPA receptor subunits

In conclusion, the importance of the present work is that neurochemically-detected increase in the expression of one or more subunits of AMPARs at synaptic level cannot directly be extrapolated in increased neurotransmission if functional evidence of facilitated synaptic responses is not obtained in parallel. More important, it should be considered that an increase in basal neurotransmission does not necessarily translate in positive functional consequences, especially if the quality of ions implicated in generation of postsynaptic EPSPs is persistently changed. Preservation of normal basal transmission could indeed allow increased strength in synaptic plasticity when required. On the other hand, the increase in GluA2/AMPARs may suggest greater availability of receptors in the intracellular pool when exposure of AMPARs is required for plasticity.

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R.C. The sponsor had no role in the design of the experimental protocols, collection and statistical analysis of the data.

Conflict of interests

At the time of experimental work Drs. L. Polenzani and M. Magnani were full-time employees of Angelini S.p.A. Dr. R. Corradetti has received research grants from Angelini S.p.A. All other Authors declare no conflict of interest.

Role of Authors

EG, RC, MM and LP participated in the design of the study and contributed intellectually to the interpretation of the data. EG, EZ and GS performed experiments and data analysis. RC supervised the project and wrote the first draft of the manuscript. All authors have contributed to and approved the final manuscript.

Legends to Figures

Fig. 1. Effect of twenty-one day treatment with trazodone or paroxetine on AMPA receptor subunit expression at hippocampal total homogenates and synapses. Western blot quantification of GluA1 and GluA2 in rat hippocampal homogenates (A) or Triton insoluble postsynaptic fractions (B) obtained from rats treated for twenty-one days with trazodone (TZD; n=5) or paroxetine (PAR; n=5), compared with saline-treated rats (n=5). Left panel: representative blot; right panel: densitometric quantification. Tubulin was used for normalization. Data are expressed as percent of the mean value of subunit expression in saline group. Columns report mean + SEM of calculated values. *p<0.05; **p<0.01 (One-way ANOVA, followed by Dunnett's multiple comparison test).

Supplementary Fig.1.

Effect of seven days treatment with trazodone and paroxetine on AMPA receptor subunit expression at hippocampal total homogenates and synapses. Western blot quantification of GluA1 and GluA2 in rat hippocampal homogenates (A) or Triton insoluble postsynaptic fractions (B) obtained from rats treated for seven days with trazodone (TZD; n=4) or paroxetine (PAR; n=4), compared with saline-treated rats (n=4). Left panel: representative blot; right panel: densitometric quantification. Tubulin was used for normalization. Data are expressed as percent of the mean value of subunit expression in saline group. Columns report mean + SEM of calculated values. One-way ANOVA indicated significant changes in the expression of both GluA1 or GluA2 were found in total homogenates ($F_{(2, 12)}$ = 0.1493, p=0.8634 and $F_{(2, 12)}$ = 3.464, p=0.0766, respectively) nor in TIF homogenates, enriched in synaptic membranes ($F_{(2,12)}$= 2.045, p=0.1852; and $F_{(2,12)}$= 3.464, p=0.0766, respectively).

Fig. 2. Typical experiment of fEPSP recording. (A) *upper panel*: Arrangement of stimulating (S1) and recording (R) electrodes on a transverse hippocampal slice. White lines indicate the surgical cut to disconnect CA3 from CA1. Lower: typical recording of fEPSP. Red trace is the average of 11 fEPSPs. Black line indicates the region for fEPSP slope measurement. Shaded area indicates the region of fEPSP voltage integral (area) measurement. (B) Recording of stimulus-response curve (SRC; left graphs) at the beginning of experiments and time-course of fEPSP responses (right graphs) upon application of the selective NMDA receptor antagonist APV (50 µM) and the cumulative addition of the selective AMPA Ca^{2+} permeable channel blocker IEM 1460 (50 µM) and the AMPA receptor antagonist NBQX (10 µM) as indicated. Upper panels show the measurements of fEPSP slope, lower panels show the corresponding measurement of fEPSP area; traces a-d in the inset are averages of 7 responses taken at times indicated by corresponding letters in the time courses. Note that the effect of APV was measurable on fEPSP area but not on fEPSP slope and that IEM 1460 in the presence of APV did not produce any effect on either parameter.

Fig. 3. Basal neurotransmission strength is not affected by twenty-one days treatment with trazodone or paroxetine. Basal stimulus-response curves of fEPSPs slope (A) and area (B) recorded at CA3/CA1 synapses in slices from rats treated with saline (*left panel*; n=11, from 7 rats), trazodone (TZD, *middle panel*; n=11, from 7 rats) or paroxetine-treated rats (PAR, *right panel*; n=9, from 6 rats) as indicated. Curves are normalized to the maximal response obtained in each preparation and scatter plots in (A) and (B) summarize the maximal fEPSP slope (MAX Slope) and area (MAX Area) obtained from stimulus-response curves. One-way ANOVA showed no differences amongst treatments for the maximal fEPSP slope (A, right plot; $F_{(2,28)}$=1.337, p=0.2788) and area (B, right plot; $F_{(2,28)}$=2.181,

p=0.1101). No differences were also found for stimulus intensity producing half-maximal response ($EStim_{50}$) and nH coefficient of stimulus-response curve slope for fEPSP slope (C) or area (D). (C) $EStim_{50}$: ($F_{(2,28)}$=0.5418, p=0.5877); nH ($F_{(2,28)}$=1.295, p=0.2897). (D) $EStim_{50}$: ($F_{(2,28)}$=0.8918, p=0.4212); nH ($F_{(2,28)}$=2.667, p=0.0870). Bars in scatter plots report mean values + SD.

Supplementary Fig. 2.

Trazodone and paroxetine do not alter fEPSPs slope and area measured at low stimulus strength. fEPSPs slope (left panel) and area (right panel) were measured at the first step of stimulation at low stimulus strength (30 μA) in slices from saline-treated animals (Saline) or in slices from trazodone (TZD) or paroxetine-treated rats (PAR). No differences were observed amongst groups of treatment in the basal neurotransmission strength for fEPSP slope ($F_{(2,28)}$=3.002, p=0.0659) and fEPSP area ($F_{(2,28)}$=1.162, p= 0.3276; One-way ANOVA). Bars in scatter plots report mean values + SD.

Fig. 4. Chronic treatment with trazodone or paroxetine did not produce the appearance of CP-AMPA receptor-mediated responses in fEPSPs. Data show the effects of *in vitro* cumulative application of APV (20 μM, 15-25 min, grey symbols) and IEM 1460 (50 μM, 20 min, in the presence of APV, black symbols) on fEPSP slope (A) and fEPSP area (B) in slices taken from rats treated for twenty-one days with saline (n= 7), trazodone (TZD n=7) or paroxetine (PAR n=7). Baseline values did not significantly differ amongst treatment groups (fEPSP slope: $F_{(2,18)}$=3.123, p=0.0685; fEPSP area: $F_{(2,18)}$=1.546, p=0.240; one-way ANOVA). (C-D) Effect of drug application (APV, grey column and APV+IEM, black column) on fEPSPs slope (C) and fEPSP area (D) of slices from twenty-one days-treated rats with saline (Saline), trazodone (TZD) or paroxetine (PAR). For each experiment, data are normalized to the respective baseline value and significance of changes was tested by one sample t test, two tails. *p<0.05; ***p<0.001.

Supplementary Fig. 3.

Basal neurotransmission strength is not affected by seven days treatment with trazodone or paroxetine in rats. Parameters of fEPSP slope (left panels) and area (right panels) are obtained from basal stimulus-response curves in slices from rats treated with saline (geen dots; n=8), trazodone (blue dots; n=7) or paroxetine (red dots; n=8). (A) maximal response; (B) stimulus intensity producing half-maximal response $EStim_{50}$; (C) nH coefficient of stimulus-response curve slope. One-way ANOVA showed no differences amongst treatments for maximal responses (fEPSP slope: $F_{(2,20)}$=0.3404, p=0.7155; fEPSP area: $F_{(2,20)}$=1.028, p=0.3758); $EStim_{50}$: (fEPSP slope: $F_{(2,20)}$=1.100, p=0.3523; fEPSP area: $F_{(2,20)}$=1.481, p=0.2512); nH: (fEPSP slope: $F_{(2,20)}$=0.9784, p=0.3932; fEPSP area: $F_{(2,20)}$=1.907, p=0.1746).

Supplementary Fig. 4

IEM-1460 inhibits EPSCs recorded from non pyramidal neurons in the CA1 region of the hippocampus. (A) *Left panel*: time-course of stimulation-evoked EPSCs recorded in whole-cell voltage clamp in control conditions and following the application of IEM-1460 (IEM; 50 μM) at time indicated by the grey area. *Right panel*: EPSCs recorded in control (Bsl) and in the presence of IEM. Traces are the average of 7 responses recorded at the times indicated by corresponding letters in (A). Calibration bars: 20 pA; 10 ms. (B) Decreases in EPSC amplitude obtained in three experiments using a similar protocol of IEM application.

Fig. 5. Effects of APV and IEM on fEPSP area. (A) *Left*: time-course plot of the effect of APV (20 μM) application on the area of fEPSPs in slices obtained after treatment for twenty-one days with saline (Saline, green circles; n=7); trazodone (TZD, blu circles; n=7) or paroxetine (PAR, red circles; n=7). Responses are normalized versus the mean value of responses during the 10 min baseline. Given are means +SEM or -SEM). *Right*: Scatter plot of the effects of APV in all groups. Symbols correspond to steady-state responses recorded the last 3 min of baseline (BSL) and APV application in each experiment. Bars report mean values + SD. (B) *Left*: time-course plot of the effect of IEM 1460 (50 μM) application in the presence of APV in the same preparations as (A). Responses are normalized versus the mean value of responses during the last 10 min of APV application. Given are means +SEM or -SEM. *Right*: Scatter plot of the effects of IEM 1460 in all groups. Symbols correspond to steady-state responses recorded the last 3 min of the application of APV or IEM 1460 (in the presence of APV: +IEM) in each experiment. Bars report mean values + SD. *$p<0.05$; **$p<0.01$ (Wilcoxon Signed Rank test, one tail). (C) Illustrative recordings in slices obtained from chronically treated rats as indicated. Traces are averages of 7 responses taken in baseline conditions (BSL) and at the end of drug application (APV or APV+ IEM). Calibration bars: 0.25 mV / 2 ms.

Legend for graphical abstract:

Chronic treatment with the antidepressants trazodone or paroxetine increases neurochemically-detected expression of GluA2 AMPAR subunit, an effect that does not translate in statistically significant changes of AMPAR-mediated neurotransmission at CA3/CA1 synapses in rats.

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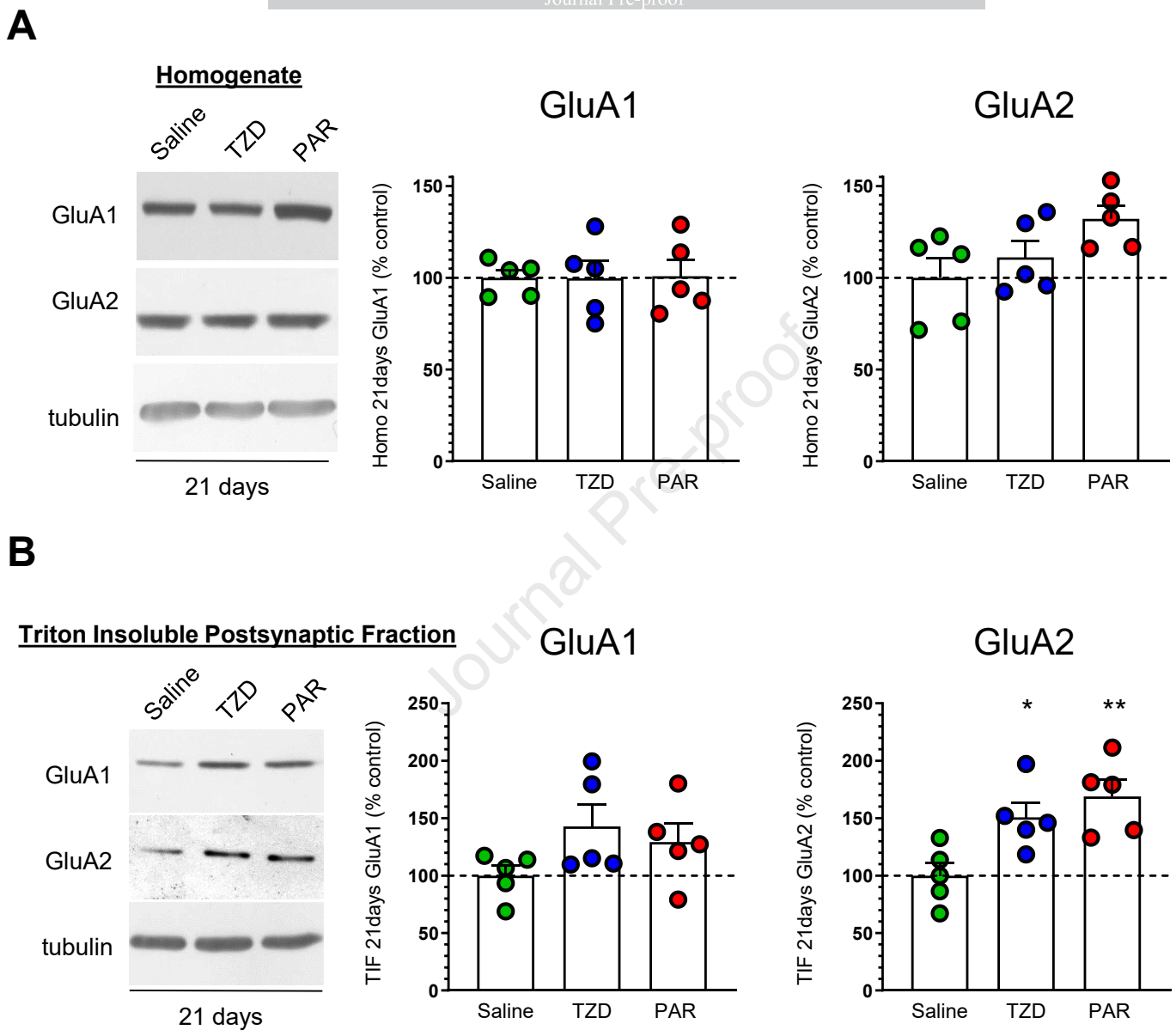

Figure 1

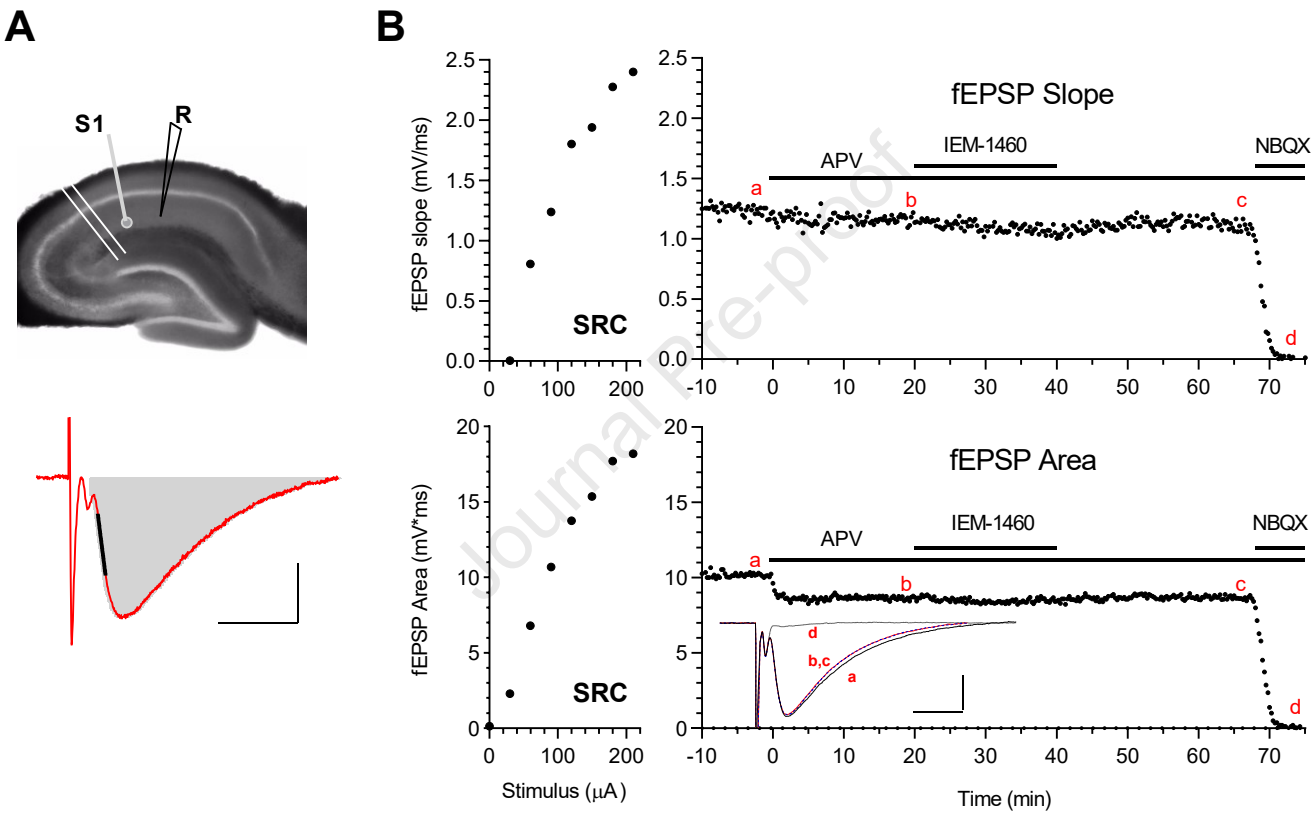

Figure 2

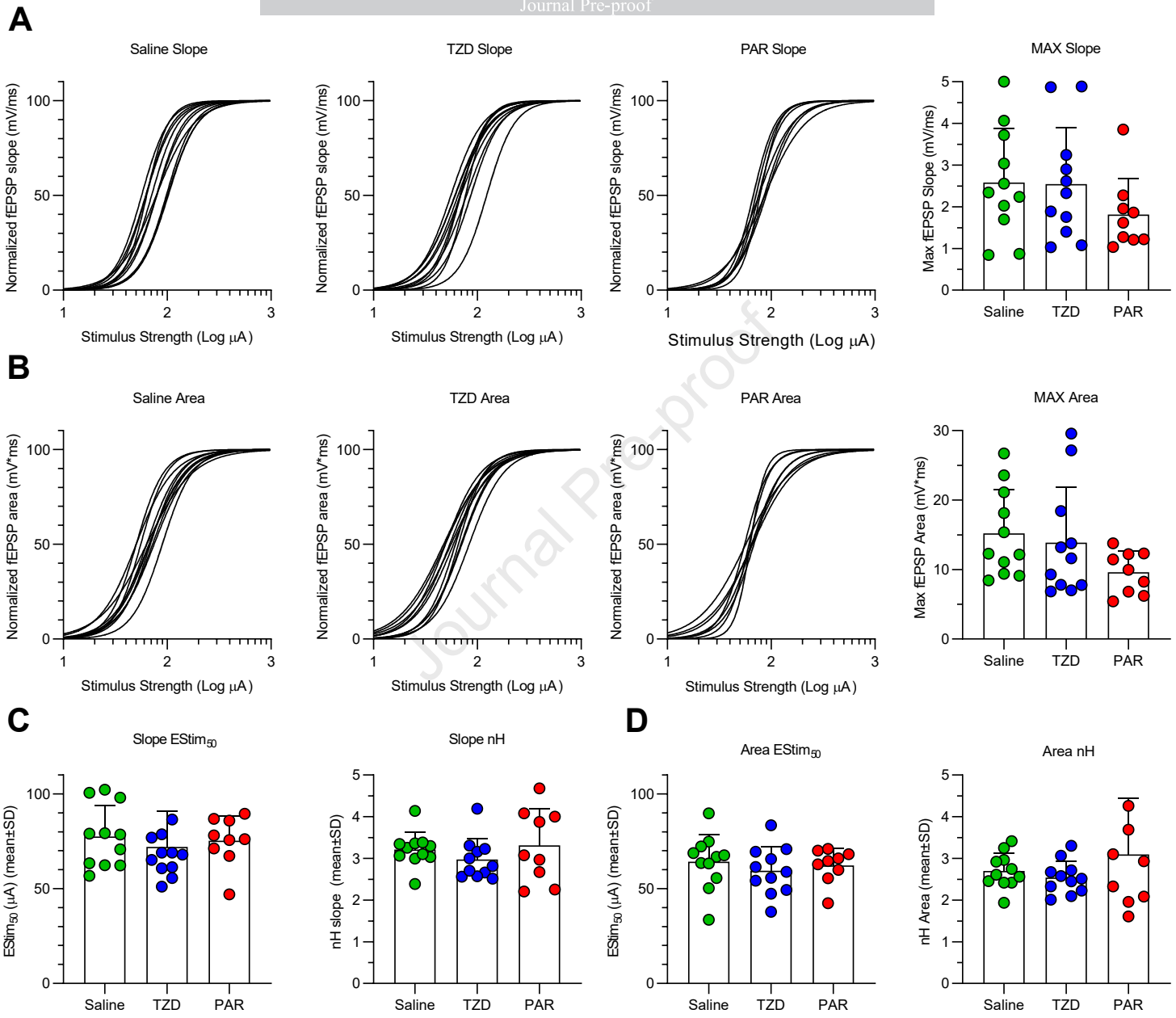

Figure 3

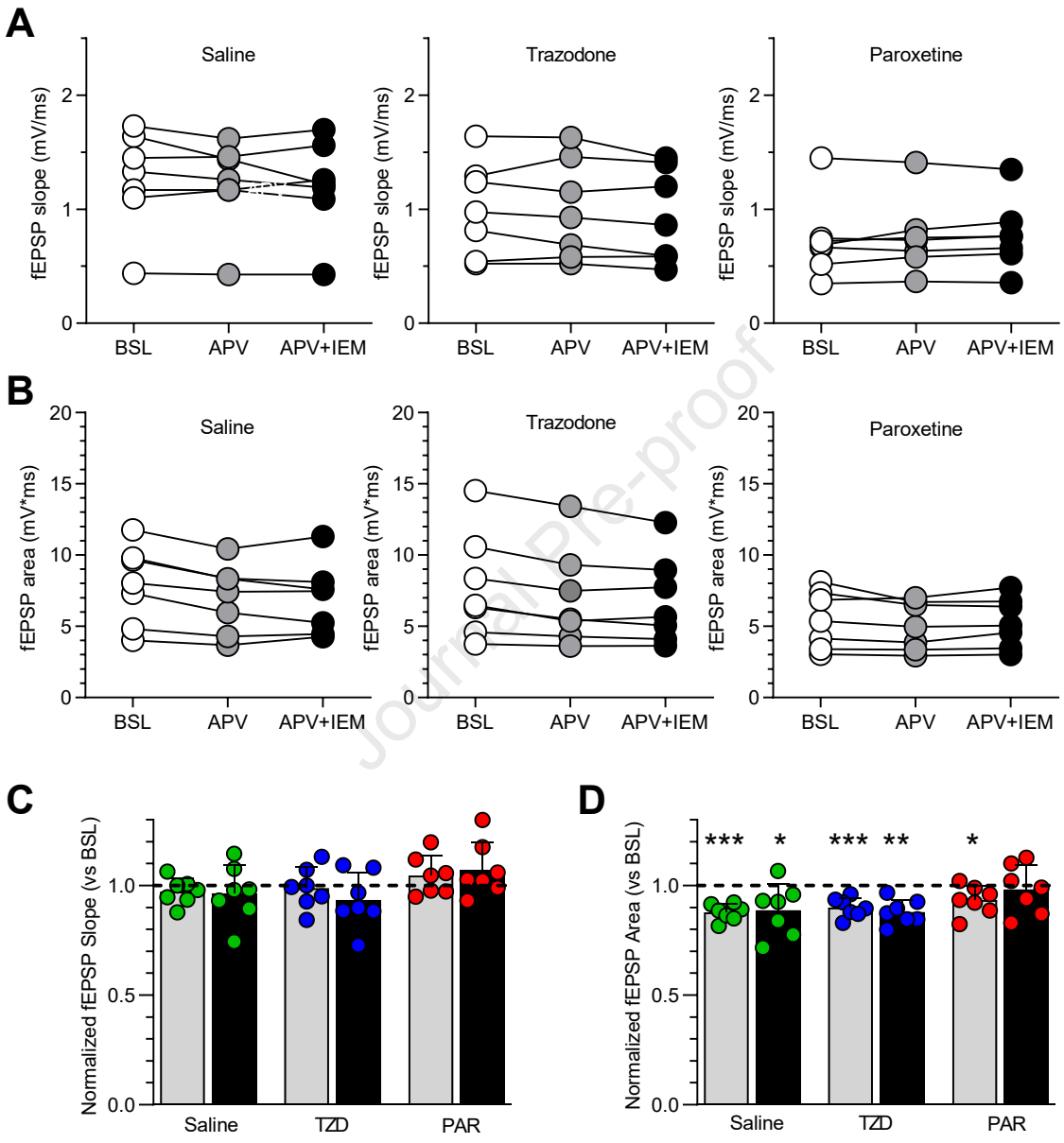

Figure 4

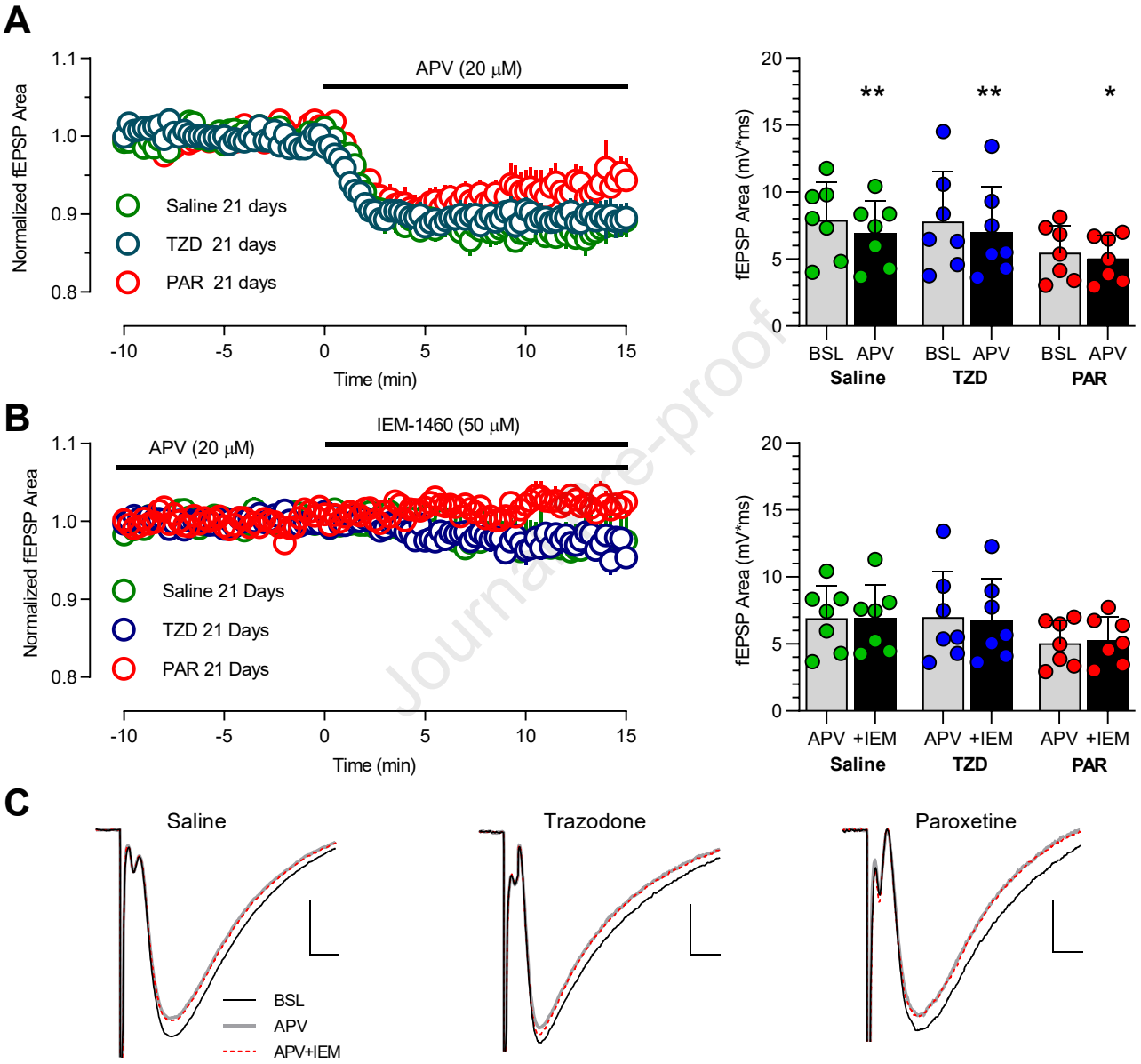

Figure 5